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Abstract

Salmonella typhimurium infection in swine causes an enterocolitis followed by a persistent carrier state, but little is known about the mechanisms that allow this organism to colonize and persist in host tissues. Neutrophils provide a first line of defense against invading pathogens such as *Salmonella typhimurium*. The purpose of this study was to evaluate porcine neutrophil function after in vivo exposure to *Salmonella* and to determine if the immunomodulator, bacillus Calmette Guerin (BCG), exerts any effect on neutrophil function or on the colonization and persistence of *S. typhimurium* in the pig. Compared to negative controls, neutrophils from pigs exposed to *S. typhimurium* exhibited significantly decreased iodination, cytochrome-C reduction, antibody-dependent cell-mediated cytotoxicity, random migration, and chemotaxis ($P \leq 0.05$). Neutrophil bactericidal activity against *S. typhimurium* was significantly enhanced. Most of the significant differences were noted in the first two days after exposure to *Salmonella*. Often the functional alterations were biphasic, peaking again 7–10 days after exposure. BCG alone significantly depressed random migration and cytochrome-C reduction in unstimulated neutrophils. The clinical course, colonization pattern, and persistence of *Salmonella* were similar between pigs receiving BCG and untreated pigs. These data suggest that *S. typhimurium* infection causes a depression in oxidative metabolism and motility, yet an increase in overall bactericidal activity against *S. typhimurium* in circulating porcine neutrophils. It also appears that BCG treatment, as reported here, does not enhance resistance of pigs to *S. typhimurium* colonization or reduce the number of persistent organisms in the porcine ileum.

Disciplines

Veterinary Infectious Diseases | Veterinary Microbiology and Immunobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

Comments

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Alteration of neutrophil function in BCG-treated and non-treated swine after exposure to *Salmonella typhimurium*

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ABSTRACT

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Salmonella typhimurium infection in swine causes an enterocolitis followed by a persistent carrier state, but little is known about the mechanisms that allow this organism to colonize and persist in host tissues. Neutrophils provide a first line of defense against invading pathogens such as *Salmonella typhimurium*. The purpose of this study was to evaluate porcine neutrophil function after in vivo exposure to *Salmonella* and to determine if the immunomodulator, bacillus Calmette Guerin (BCG), exerts any effect on neutrophil function or on the colonization and persistence of *S. typhimurium* in the pig. Compared to negative controls, neutrophils from pigs exposed to *S. typhimurium* exhibited significantly decreased iodination, cytochrome-C reduction, antibody-dependent cell-mediated cytotoxicity, random migration, and chemotaxis ($P \leq 0.05$). Neutrophil bactericidal activity against *S. typhimurium* was significantly enhanced. Most of the significant differences were noted in the first two days after exposure to *Salmonella*. Often the functional alterations were biphasic, peaking again 7–10 days after exposure. BCG alone significantly depressed random migration and cytochrome-C reduction in unstimulated neutrophils. The clinical course, colonization pattern, and persistence of *Salmonella* were similar between pigs receiving BCG and untreated pigs. These data suggest that *S. typhimurium* infection causes a depression in oxidative metabolism and motility, yet an increase in overall bactericidal activity against *S. typhimurium* in circulating porcine neutrophils. It also appears that BCG treatment, as reported here, does not enhance resistance of pigs to *S. typhimurium* colonization or reduce the number of persistent organisms in the porcine ileum.

ABBREVIATIONS

ADCC, antibody-dependent, cell-mediated cytotoxicity; BCG, bacillus Calmette Guerin; PBS, phosphate-buffered saline; WBC, white blood cells.

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INTRODUCTION

Salmonella typhimurium causes enterocolitis in swine and can persist within the intestinal wall and associated lymph nodes for at least several months (Wood et al., 1989). *Salmonella typhimurium* is also a leading cause of food-borne illness in humans (Todd, 1989). The ability of infected swine to persist in a subclinical carrier state, yet shed large numbers of *Salmonella* during periods of stress (Hansen et al., 1964; Williams and Newell, 1970; Morgan et al., 1987) makes them an important reservoir of infection.

Immune mechanisms involved in the establishment and maintenance of the *S. typhimurium* carrier state in swine are not clear. Most studies describing immunity to *Salmonella* have focused on other animal species and the role of the macrophage in resistance to *Salmonella* (Lowrie et al., 1979; Buchmeier and Heffron, 1989; Hatchigian et al., 1989). The neutrophil, however, is an important mediator of host defense, especially in early stages of infection. Smith et al. (1981a,b, 1985) have described alterations in porcine neutrophil adhesion, chemotaxis, and bactericidal activity after exposure to *S. typhimurium*, but no confirming reports are available. To our knowledge, there are no published reports describing the effect of *S. typhimurium* infection on the respiratory burst or cytotoxic activity of porcine neutrophils.

Biologic response modifiers have been proposed as a means of enhancing nonspecific immunity to bacterial infections. Studies have shown that bacillus Calmette-Guerin (BCG) administration increases the LD₅₀ of *S. typhimurium* in mice (Senterfitt and Shands, 1970; Yarkoni and Bekierkunst, 1976). Bestatin treatment in mice reduces persistent populations of *S. typhimurium* in the liver and spleen (Dickneite et al., 1984). To our knowledge, there are no reports of similar studies in swine.

In this study, we characterized eight parameters of neutrophil function during acute and convalescent stages of *S. typhimurium* infection in swine. We also characterized the effect of prior BCG administration on neutrophil function in *Salmonella*-free and -infected swine. We measured the influence of BCG on the carrier state by comparing persistent *Salmonella* populations in BCG-treated and nontreated swine.

MATERIALS AND METHODS

Pigs

Twenty-two hysterectomy-derived, colostrum-deprived pigs of both sexes were raised in isolation to 8 weeks of age. Periodic bacteriologic examinations of rectal swabs and feces were performed as described previously (Wood et al., 1989). All pre-exposure samples were negative for *Salmonella*.

Bacteria

Salmonella typhimurium strain 798 (Wood et al., 1989), originally isolated from a clinical case of porcine salmonellosis, was used throughout this study. Static cultures used for pig exposure were grown overnight in Luria broth at 37°C and contained approximately 1.5×10^9 cfu ml⁻¹. Bacteria for the bactericidal assay were grown to mid-log phase in Luria broth at 37°C in a shaking incubator (150 rpm). The bacteria were then centrifuged at $2500 \times g$ for 15 min. The pellet was washed in 0.01 M phosphate-buffered saline (PBS), recentrifuged, and resuspended in tryptose soy broth at 1/20 original volume. Aliquots for the assay were stored at -70°C.

BCG (Calbiochem, San Diego, CA) was reconstituted in PBS (2 mg ml⁻¹) and killed by autoclaving. The killed suspension was mixed 1:1 with Freund's incomplete adjuvant and stored at 4°C prior to use.

Exposure of pigs

The pigs were divided into four isolated groups. Group 1 ($n=6$) received BCG and *Salmonella*, group 2 ($n=6$) received only *Salmonella*, group 3 ($n=5$) received only BCG, and group 4 ($n=5$) served as a nontreated, non-infected control according to the following schedule.

Each pig received two subcutaneous injections, 15 days apart (days -17 and -2), of either 2 ml BCG in Freund's incomplete adjuvant or 2 ml sterile saline. Two days after the second injection (day 0), each pig received a 10-ml oral dose of either *S. typhimurium* or sterile culture medium.

Evaluation of clinical response

Rectal temperatures were obtained on days -5 to -3 and days -1 to 7. Rectal swabs were obtained from each pig on days 1-4. Fecal samples were collected weekly throughout the study. Total white blood cell (WBC) counts were performed on each day that neutrophil assays were run (see below).

Collection of blood and separation of neutrophils

Each pig was bled via the cranial vena cava 1 and 3 days prior to the first subcutaneous injection and on Days 1, 2, 3, 4, 7, 10 and 14 after exposure to *S. typhimurium* or culture medium. Thirty-five milliliters of blood were drawn into syringes preloaded with 1 ml 7.5% EDTA in PBS. Most of the erythrocytes were removed by hypotonic lysis. The remaining cells were centrifuged through a Ficoll-diatrizoate density gradient. The neutrophil-rich pellet was harvested, and residual erythrocytes were removed by an additional hypotonic lysis step. The remaining cells were washed and resuspended in PBS and

adjusted to 5×10^7 cells ml^{-1} . The purity of the neutrophil preparation, as determined by microscopic morphologic examination, was $> 90\%$ granulocytes; viability, as determined by trypan blue exclusion, was $> 95\%$.

Assays of neutrophil function

The following assays of neutrophil function were performed: antibody-dependent, cell-mediated cytotoxicity (ADCC), iodination, *Staphylococcus aureus* ingestion, stimulated and unstimulated cytochrome-C reduction, random migration, chemotaxis, and an MTT [(3-,4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide] bactericidal assay.

The *S. aureus* ingestion and iodination assays were performed as described by Roof and Kramer (1989) except that the harvested neutrophils were not incubated with *Salmonella*.

The cytochrome-C reduction assay was performed using a modification of the procedure described by Roth et al. (1986). Neutrophils were incubated with cytochrome-C containing (stimulated) or without (unstimulated) opsonized zymosan for 10 min prior to reading the optical density of the solution at 550–650 nm.

Random migration under agarose was performed as described by Roth and Kaeberle (1981a), except that the agarose contained M199 medium with Hank's salts and porcine serum, and the plates were fixed with 8% glutaraldehyde. The area of migration in square millimeters was calculated from the measured radius of migration.

The chemotaxis assay was performed in a manner similar to that described by Smith et al. (1985) with the following modifications: using the same agarose mixture as described above, 2 mm diameter wells, spaced 3.5 mm apart, were loaded with $7.5 \mu\text{l}$ (3.75×10^5) neutrophils. After incubating for 3 h, the plates were fixed and stained as described for random migration plates. Results were reported as the chemotactic index, the distance migrated towards the chemoattractant (zymosan-activated serum) divided by the distance migrated toward the control.

Antibody-dependent cell-mediated cytotoxicity was measured by a chromium-release assay as described by Roth and Kaeberle (1981b) except that the supernatant was harvested from 96-well plates using a SCS harvesting system (Skatron, Sterling, VA). The results were expressed as percent specific release of ^{51}Cr .

The MTT bactericidal assay was performed as described by Stevens et al. (1991) except for the following modification: the bacteria were adjusted to a concentration of 2.5×10^8 cfu/ml RPMI-1640 containing 5% porcine or rabbit immune serum and 2.5% fresh normal porcine serum (complement source) and were incubated for 30 min at room temperature. Neutrophils were adjusted to 1×10^7 cells ml^{-1} in PBS. Neutrophils and opsonized bacte-

ria were incubated together for 60 or 90 min. Controls included wells receiving only neutrophils and wells receiving only PBS. After the addition of isopropanol, the plates were tightly covered and stored overnight. The optical densities were read in a microplate reader at 570–630 nm (Dynatec, Torrance, CA).

Results of the MTT bactericidal assay were expressed as percent killing by neutrophils. The average of quadruplicate wells was calculated for each sample and the background due to neutrophil debris was subtracted from each average. A linear regression was performed on the values of the wells containing the known reductions in bacterial cell number, and a best-fitting line was obtained. Corrected experimental averages were entered into the equation of the best-fitting line to calculate the percent of bacteria killed by the neutrophils. Each plate was compared to its own standard curve to account for day-to-day and plate-to-plate variations.

Necropsy

Two pigs from each group were necropsied on Days 8, 15 and 22 with the exception of Groups 3 and 4 on Day 8 where only one pig was necropsied from each group. The pigs were anesthetized, exsanguinated, and necropsied as described by Wood et al. (1991). The following tissues were collected aseptically from *Salmonella*-infected pigs: spleen, liver, ileocolic lymph node, terminal ileum, apex of cecum and peritoneal fluid. The spleen, terminal ileum and apex of cecum were sampled from the pigs not receiving *Salmonella*.

Bacteriologic examination

Liver, ileocolic lymph nodes, cecum, peritoneal fluid, rectal swabs and feces were examined qualitatively for the presence of *Salmonella* as described by Wood et al. (1989). Quantitative estimations of *S. typhimurium* populations in the spleen and ileum were determined by a most probable number method (Wood and Rose, 1992).

Statistical analysis

The study was divided into four periods: period 1, pre-exposure; period 2, Days 1 and 2 after exposure to *Salmonella*; period 3, Days 3 and 4 after exposure; period 4, Days 7, 10 and 14 after exposure. Analyses of variance were performed for each assay, using average values from each animal in each period. The data were blocked by date to minimize the effect of daily variation inherent in these assays. *P*-values were considered significant at the 0.05 level.

RESULTS

The clinical response was similar in groups receiving *Salmonella* with or without BCG and was characterized by soft feces and a mild, often biphasic, febrile response (data not shown). Noninfected pigs receiving BCG alone showed no systemic clinical signs of illness. All groups, except the noninfected, nontreated controls, demonstrated an increase in WBC count immediately after treatment with BCG and/or *Salmonella* (Fig. 1, Day 1). Those pigs receiving *Salmonella* with or without BCG usually demonstrated a second leukocytosis 7–10 days postinfection. However, because the timing of the second leukocytosis was variable among individual pigs, the group averages shown in Fig. 1 do not reflect this trend.

Neutrophils from pigs receiving *Salmonella* and/or BCG exhibited a general depression in ADCC activity (Fig. 2) compared to control animals. ADCC activity was significantly reduced ($P \leq 0.05$) in both groups receiving *Salmonella* during periods 2 and 4 and in pigs receiving only BCG during period 4.

Cytochrome-C reduction was significantly decreased ($P \leq 0.05$) in unstimulated (Fig. 3(a)) and stimulated (Fig. 3(b)) neutrophils from all three infected and/or treated groups in period 2 when compared to controls. How-

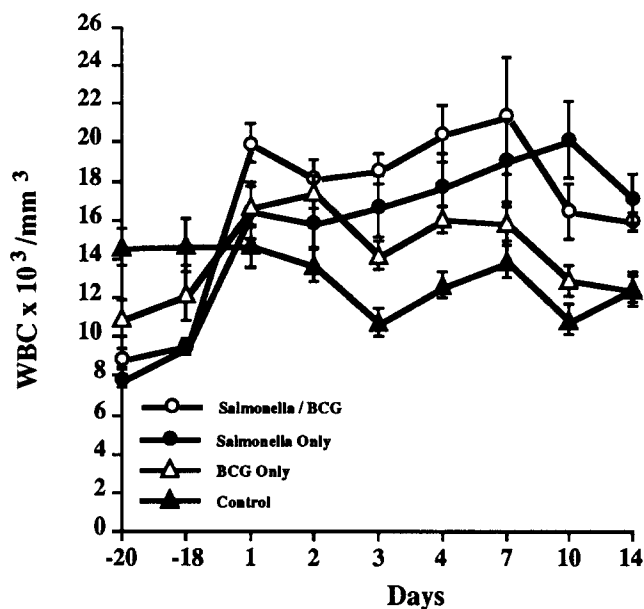


Fig. 1. White blood cell counts (mean \pm SEM) in pigs receiving *S. typhimurium* and/or BCG. *Salmonella* was given on day 0. BCG was given on Days -17 and -2.

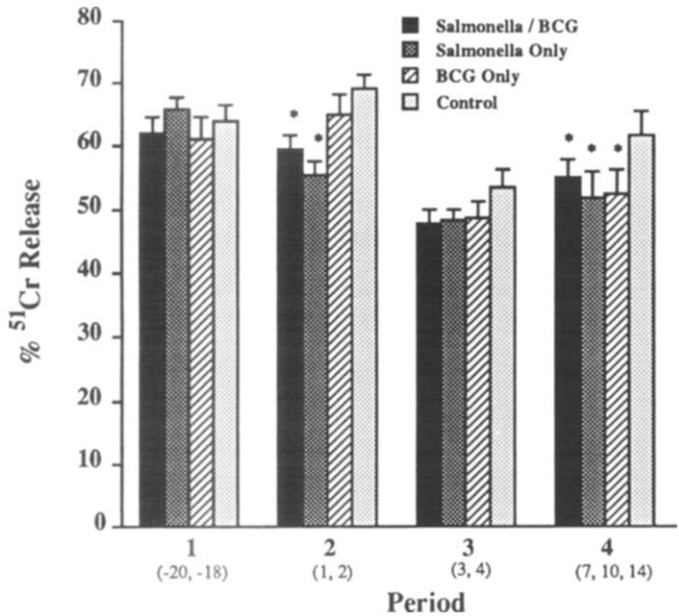


Fig. 2. Antibody-dependent cell-mediated cytotoxicity (mean ± SEM) of neutrophils from pigs receiving BCG (Days -17 and -2) and/or *S. typhimurium* (day 0). Periods reflect average of values from days indicated in parentheses. *, different from controls ($P \leq 0.05$).

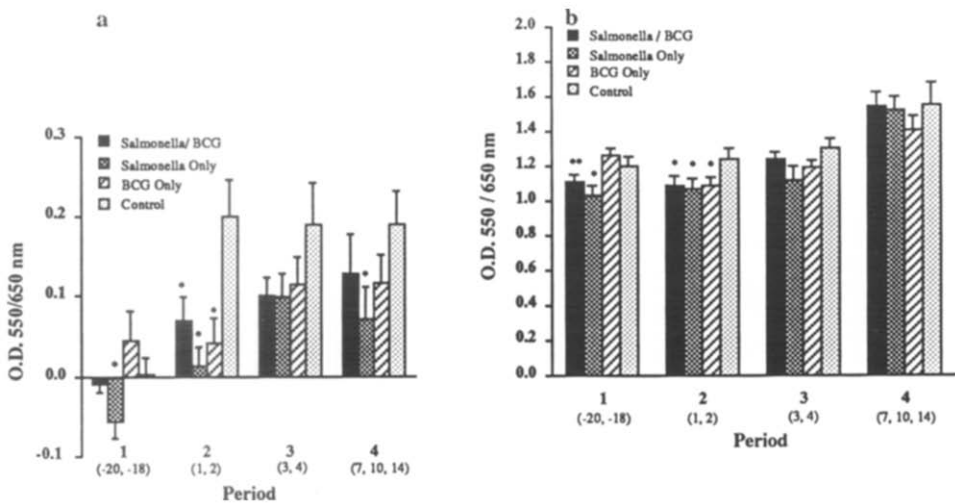


Fig. 3. Cytochrome-C reduction (mean ± SEM) by unstimulated (a) and zymosan-stimulated (b) neutrophils from pigs receiving BCG (Days -17 and -2) and/or *S. typhimurium* (Day 0). Periods reflect average of values from days indicated in parentheses. *, different from controls ($P \leq 0.05$). **, different from group receiving BCG only ($P \leq 0.05$).

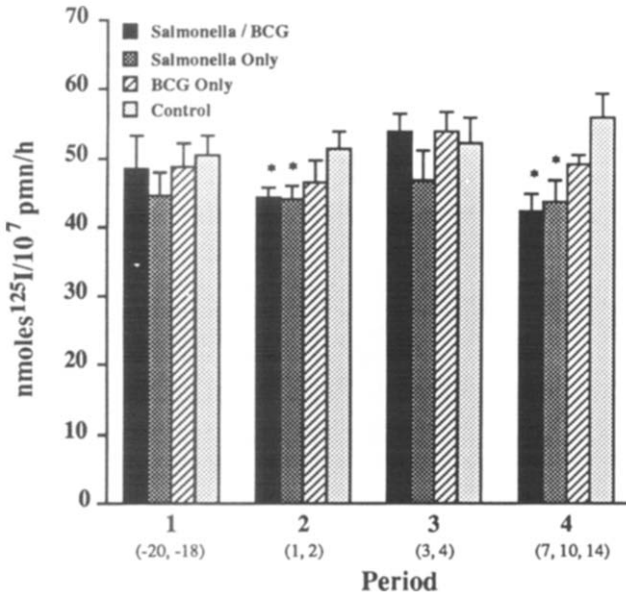


Fig. 4. Iodination activity (mean \pm SEM) of porcine neutrophils. Pigs received BCG on Days -17 and -2 . *Salmonella* was given on Day 0. Periods reflect average of values from days indicated in parentheses. *, different from controls ($P \leq 0.05$).

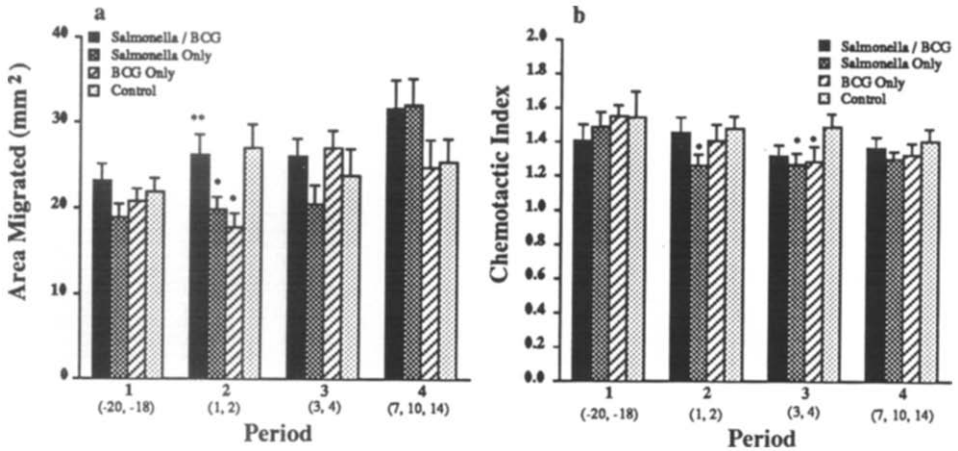


Fig. 5. Random migration (a) and chemotaxis (b) (mean \pm SEM) of neutrophils from pigs receiving BCG (days -17 and -2) and/or *S. typhimurium* (Day 0). Periods reflect average of values from days indicated in parentheses. *, different from controls ($P \leq 0.05$); **, different from groups receiving *Salmonella* only or BCG only ($P \leq 0.05$); ***, different from group receiving *Salmonella* only ($P \leq 0.05$).

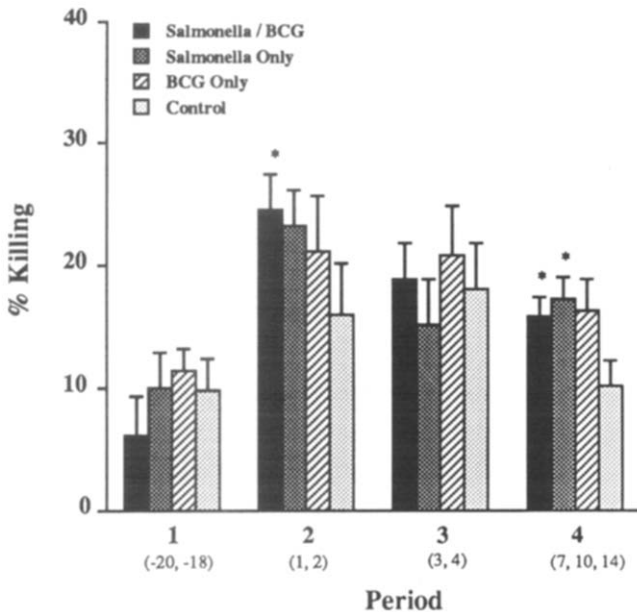


Fig. 6. Bactericidal activity (mean \pm SEM) of porcine neutrophils against *S. typhimurium* pre-opsionized with rabbit antibody and complement. Neutrophils and bacteria were incubated together for 60 min. Pigs received BCG on Days -17 and -2. *Salmonella* was given on Day 0. Periods reflect average of values from days indicated in parentheses. *, different from controls ($P \leq 0.05$).

ever, a significant difference ($P \leq 0.05$) was also detected between the group receiving only *Salmonella* and the control group before exposure (period 1).

Iodination was significantly reduced ($P \leq 0.05$) in groups receiving *Salmonella* during periods 2 and 4 (Fig. 4) when compared to controls in the same period. There was no statistically significant ($P \leq 0.05$) depression in the group receiving only BCG.

Neutrophil motility was inhibited in pigs receiving only *Salmonella* and in pigs receiving only BCG compared to controls. Random migration (Fig. 5(a)) was significantly decreased ($P \leq 0.05$) in these groups during period 2. The chemotactic index (Fig. 5(b)) was significantly depressed ($P \leq 0.05$) in pigs receiving only *Salmonella* during period 2 and in pigs receiving either *Salmonella* or BCG during period 3.

Neutrophil bactericidal activity against *S. typhimurium* was increased ($P \leq 0.05$) after exposure to *Salmonella* (Figs. 6 and 7). Assay results are shown using rabbit or pig serum as an opsonin with 60- or 90-min incubation periods, respectively. The original optimization of this assay was performed using neutrophils from random adult swine, and satisfactory killing rates were obtained with rabbit serum and a 60-min incubation. The pigs in this study,

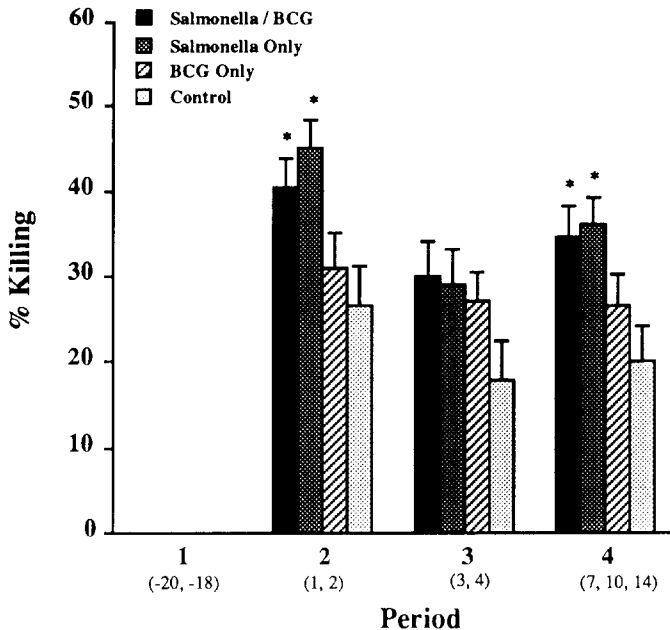


Fig. 7. Bactericidal activity (mean \pm SEM) of porcine neutrophils against *S. typhimurium* preopsonized with porcine antibody and complement. Neutrophils and bacteria were incubated together for 90 min. Pigs received BCG on Days -17 and -2 . *Salmonella* was given on Day 0. Periods reflect average of values from days indicated in parentheses. *, different from controls ($P \leq 0.05$).

however, had low killing rates using this protocol (Fig. 6, period 1). In the time interval between periods 1 and 2, a suitable immune pig serum source became available and various incubation intervals were assessed. For the remaining periods in this study, simultaneous assays using pig serum and a 90-min incubation interval, which were believed to yield more sensitive results, were performed along with assays using the original protocol. Both sets of data show similar overall trends.

Salmonella infection and BCG administration had no significant effects on *S. aureus* ingestion by neutrophils (data not shown).

Infected pigs receiving BCG and nontreated infected pigs had similar patterns of *S. typhimurium* colonization and persistence in internal organs. Rectal swabs were culture-positive for *Salmonella* 24 h after exposure in all but one pig, and with the exception of one different pig each day, rectal swabs remained positive throughout the period of collection. The ileocolic lymph nodes, ileum, and cecum were infected in all exposed animals at necropsy. *Salmonella* was cleared from the spleen by Day 8 post-exposure in three of four pigs necropsied. From one pig in the *Salmonella*/BCG group, less than 5 organisms per 10 g spleen were recovered. *Salmonella* was recovered from

the liver of all pigs necropsied on Day 8. By Day 15, we recovered *Salmonella* from one out of four livers. Both organs were culture-negative in all pigs necropsied on Day 22. We did not culture *Salmonella* from the peritoneal fluid of any animal at any necropsy. Feces from *Salmonella*-infected pigs were culture-positive in all post-exposure samples. Feces from pigs not receiving *S. typhimurium* did not contain *Salmonella*.

Populations of *S. typhimurium* in the ileal wall were similar between the BCG-treated and nontreated infected groups (data not shown). The numbers of bacteria isolated were fairly stable throughout the study, the \log_{10} of the population averaging 6.0 on Day 8 and 6.7 on Day 22.

DISCUSSION

Functional changes in circulating neutrophils occur in swine after oral exposure to *S. typhimurium*. Iodination and ADCC were depressed in infected pigs compared to controls immediately after infection (period 2). Activity normalized 3–4 days after infection (period 3), but was depressed again 7–14 days after infection (period 4). A waxing and waning clinical nature of *S. typhimurium* infection in swine has been previously described (Wilcock, 1986). Our study demonstrates that alterations in certain neutrophil functions follow a similar pattern.

Cytochrome-C reduction was also depressed in infected pigs during period 2. The significance of this depression in the group receiving only *Salmonella* must be interpreted with caution, as pre-exposure assays from this group were also significantly different from controls. The depression evident in the *Salmonella*/BCG group during period 2, however, is considered to be a result of the treatment protocol.

Both cytochrome-C reduction and iodination measure oxygen-dependent bactericidal activities of the neutrophil. Alteration of neutrophil oxidative metabolism is a common pathogenic mechanism among many infectious agents. Depression of the oxidative burst has been demonstrated in bovine neutrophils after infection with bovine viral diarrhea virus (Roth et al., 1986), parainfluenza virus (Briggs et al., 1988), and *Brucella abortus* (Canning et al., 1988).

Salmonella typhimurium infection enhances the bactericidal activity of porcine neutrophils against *S. typhimurium*. This enhancement was evident throughout the duration of the experiment (to 15 days after infection), especially in the assay using porcine serum. These findings are in partial agreement with those of Smith et al. (1981b), who reported increased porcine neutrophil bactericidal activity against *Staphylococcus aureus* 48 h after infection with *S. typhimurium* but detected no increases at 96 h or 6 days.

It seems paradoxical that neutrophil bactericidal activity should be increased when parameters of neutrophil oxidative metabolism are depressed.

Oxidative bactericidal mechanisms are considered more potent than non-oxidative mechanisms (Smolen, 1989). Our data suggest a possible role for non-oxidative killing of *S. typhimurium* by porcine neutrophils. Cationic proteins, which comprise the major portion of neutrophil and macrophage cytoplasmic granule contents, have non-oxidative bactericidal action on *S. typhimurium* in other animal species (Shafer et al., 1984; Stinavage et al., 1989; Groisman and Saier, 1990) and may be important in swine. Alternatively, oxidative bactericidal mechanisms not evaluated by cytochrome-C reduction or iodination may be enhanced. Similar apparently conflicting data were reported by Eisenstein et al. (1988), wherein vaccination of mice with *aroA* mutants of *S. typhimurium* provided good immunity despite depression of specific macrophage functions.

Salmonella typhimurium infection reduced neutrophil motility immediately after infection. Both random migration and chemotaxis were inhibited during period 2. Inhibition of chemotaxis continued through Days 3 and 4 post-infection (period 3). These findings support those of Smith et al. (1985), who reported decreased chemotaxis at 72 h after infection. Smith et al., however, did not detect an alteration in chemotaxis 24 h after infection. Other infectious agents, such as *Bacteroides* spp. (Adamu and Sperry, 1981) or infectious bovine rhinotracheitis virus (Briggs et al., 1988), decrease chemotaxis of neutrophils in other animal species.

BCG has been used as a non-specific immunoenhancer in the treatment of human malignancies (Crispen, 1986). Its effects on the immunomodulation of bacterial infections have been investigated in mice. BCG or its purified components increase murine resistance to *S. typhimurium* (Senterfitt and Shands, 1970), *Klebsiella pneumoniae*, *Escherichia coli*, and *Streptococcus pneumoniae* (Fogler, 1984).

In our study, BCG had no statistically significant main effect on any of the neutrophil function parameters examined. That is, the average of both groups receiving BCG was not statistically different ($P > 0.05$) from the average of groups not receiving BCG.

The effects of BCG observed in this study differ from those seen in murine macrophages treated with BCG (Fogler, 1984). While decreased migration was observed in both studies, treated murine macrophages exhibited increased oxidative metabolism and ingestion in contrast to the decreased oxidative burst and unaltered ingestion seen in our treated porcine neutrophils.

Overall, BCG treatment had few significant effects on normal porcine neutrophil function and did not reverse the effects of *Salmonella*-induced neutrophil functional depression. It did not further enhance the increased bactericidal activity detected in *Salmonella*-infected pigs. BCG did not alter the porcine clinical response to *S. typhimurium* nor did it affect colonization of and persistence of *S. typhimurium* in internal organs of pigs. It appears, therefore, that although BCG may exert minor influences on neutrophil function,

it is not an effective immunomodulator of *S. typhimurium* infections in swine when used as reported here.

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